

## Histochemical characterisation of oocytes of the swordfish *Xiphias gladius*

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**SUMMARY:** This paper reports a histological, histochemical and immunohistochemical characterisation of growing oocytes of the swordfish *Xiphias gladius*. The presence and distribution of carbohydrates, proteins, lipids, calcium, iron, vitellogenin/Vg, zona radiata protein/Zrp, metallothionein/Mt, and thyroid hormones/T<sub>3</sub>-T<sub>4</sub> were studied during oogenesis (cortical alveoli, globules, yolk-granules, cytoplasm, follicular and radiata envelopes). During the initial vitellogenic phase, the oocytes showed cortical alveoli and oil globules containing neutral lipids exclusively. During this phase, small yolk granules appeared around the peripheral cytoplasm, and they increased through exogenous vitellogenesis. Yolk granules were composed of glycoproteins, calcium, iron, and proteins rich in lysine, arginine, tyrosine, tryptophan, cysteine and cystine. Vg and Mt were immunohistochemically detected in yolk. The follicular envelope contained proteins rich in amino acids. Moreover, calcium and thyroid hormones (triiodotyronine and thyroxine/T<sub>3</sub>, T<sub>4</sub>) were detected in this cell envelope. Cortical alveoli, which contained carboxylated and neutral glycoconjugates, were especially rich in N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, galactose and sialic acid. Finally, the zona radiata was mainly proteinaceous in nature and was composed of calcium and neutral glycoproteins. The egg envelope or chorion and the liver showed specific immunoreactivities by using anti-salmon Zrp as the primary antiserum.

**Keywords:** histochemistry, carbohydrates, lipids, glycoconjugates, vitellogenin, zona radiata protein, metallothionein, thyroid hormones, cations, oocytes, *Xiphias gladius*.

**RESUMEN:** CARACTERIZACIÓN HISTOQUÍMICA DE OVOCITOS DEL PEZ ESPADA, *XIPHIAS GLADIUS*. – En este trabajo se realiza una caracterización histológica, histoquímica e inmunohistoquímica de los ovocitos en crecimiento del pez espada, *Xiphias gladius*. Se estudia la presencia y distribución de carbohidratos, proteínas, lípidos, calcio, hierro, vitelogenina/Vg, proteína radiada/Zrp, metalotioneina/Mt y hormonas tiroideas/ T<sub>3</sub>-T<sub>4</sub> durante la ovogénesis (alveolos corticales, glóbulos, gránulos-vitelo, citoplasma, capas folicular y radiada). Durante la fase inicial de la vitelogénesis, los ovocitos presentan alveolos corticales y una gota lipídica conteniendo exclusivamente lípidos neutros. Durante esta fase en la periferia del citoplasma aparecen pequeños granulos de vitelo, incrementando progresivamente durante la vitelogénesis exógena. Los gránulos de vitelo están compuestos por glicoproteínas, calcio, hierro, proteínas ricas en lisina, arginina, tirosina, triptófano, cisteína y cistina, y contienen Vg y Mt. La capa folicular de los ovocitos del pez espada está compuesta por glicoproteínas y proteínas ricas en diferentes aminoácidos. Esta envuelta contiene calcio y hormonas tiroideas/ T<sub>3</sub> y T<sub>4</sub>. Los alveolos corticales están constituidos por glicoconjugados carboxilados y neutros, y son especialmente ricos en N-acetil-D-galactosamina, N-acetil-D-glucosamina, galactosa y ácido siálico. Finalmente, la capa radiada, fundamentalmente proteica, contiene calcio y glicoproteínas neutras. Utilizando anti-salmon Zrp, como anticuerpo primario, el corion o capa radiada y el hígado muestran una inmunoreactividad específica.

**Palabras clave:** histoquímica, carbohidratos, lípidos, glicoconjugados, vitelogenina, proteína radiada, metalotioneinas, hormonas tiroideas, cationes, ovocitos, *Xiphias gladius*.

## INTRODUCTION

During the reproductive cycle of fish species, the chemical composition of the fish oocytes (cytoplasm, cortical alveoli, yolk, zona radiata, granulosa and theca layers) and the mobilisation of macromolecules and cations between the liver and gonads have been studied by several authors (Wallace, 1978; Khoo, 1979; Gutiérrez *et al.*, 1985; Selman and Wallace, 1986; Mayer *et al.*, 1988; González de Canales *et al.*, 1992; Sarasquete *et al.*, 1993; Muñoz-Cueto *et al.*, 1996; Grau *et al.*, 1996; Corriero *et al.*, 2004). However, the histochemical localisation of specific metal-binding-proteins (metallothionein/Mt), cations (calcium, iron), thyroid hormones and specific lectin-glycoconjugates during fish oogenesis has received less attention (Sarasquete *et al.*, 2002 a,b; Motta *et al.*, 2005).

Cytological analysis has revealed that fish oogenesis is characterised by the appearance of membrane-limited round structures known as the cortical alveoli. During vitellogenesis, the alveoli are gradually displaced to the oocyte cortex, due to the centripetal storage of yolk (Gutiérrez *et al.*, 1985; Selman and Wallace, 1986; Motta *et al.*, 2005). At fertilisation, alveoli polysialoglycoprotein content is proteolytically cleaved, leading to the formation of specific glycopeptides (L-hyosopporin) which are released into the perivitelline space (Inoue and Inoue, 1986; Seko *et al.*, 1989), and this contributes to the transformation of the vitelline envelope into the fertilisation membrane (Kudo and Teshima, 1998). The presence of high molecular mass polysialoglycoproteins or H-hyosopporins has been reported in *Fundulus heteroclitus* (Selman *et al.*, 1986), in medaka, *Oryzias latipes* (Kitajima *et al.*, 1989) and in several salmonids (Inoue *et al.*, 1987; Kitajima *et al.*, 1988). Glycoconjugates may be involved in binding of hormones; in bacteria agglutination; in the transport of metabolites and ions across the plasmalemma; in sperm-egg binding; and in polyspermic inhibition (Prokop *et al.*, 1974; Miller and Ax, 1990; Skutelsky *et al.*, 1994; Motta *et al.*, 2005).

In teleost fish species, oocyte growth and yolk protein formation is mainly due to the hormonal regulation of hepatic synthesis and ovarian uptake of exogenous proteins, such as vitellogenin (Vg) and zona radiata proteins (Zrp) (Wallace, 1985; Hamazaki *et al.*, 1987; Hyllner and Haux, 1992; Celius and Walter, 1998). Vg is a complex glycolipophosphoprotein (300–640 kDa) thought to be a heterodimer or to have multiple forms (Mommensen and Walsh, 1988; Walther,

1993; Tyler and Sumpter, 1996; Hiramatsu *et al.*, 2006). In the egg, Vg undergoes proteolytic cleavage into yolk proteins, the lipid-rich lipovitellins, highly phosphorylated phosvitins and a  $\beta'$ -component (Hiramatsu *et al.*, 2001 and Romano *et al.*, 2004), which are the major source of nutrients for eggs and developing embryos. In plasma, Vg binds  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{K}^{+}$  to provide minerals to the developing fish (Palumbo *et al.*, 2007). Additionally, the primary degradation products of Vg have also been shown to play a role in regulating oocyte hydration and buoyancy of teleostean eggs (Matsubara *et al.*, 1999).

In vertebrates, the egg envelope, referred to as the zona pellucida in mammalian eggs, is a fibrous and noncollagenous extracellular matrix surrounding the eggs and composed of three to four homologous glycoproteins with a common ZP domain. While ZP1 and ZP3 are present in all vertebrate species, ZPX is not found in mammals and ZP2 is not found in zona radiata of fish eggs (Waclawek *et al.*, 1988; Hamazaki *et al.*, 1989; Yamaguchi *et al.*, 1989; Hyllner *et al.*, 1994; Epifano *et al.*, 1995; Modig *et al.*, 2006). In fish species, proteins of the zona radiata are synthesised in either the ovary or the liver (Hyllner *et al.*, 2001; Hamazaki *et al.*, 1987). The precursors of these proteins have been identified as choriogenin H (high molecular weight) and choriogenin L (low molecular weight), respectively (Lee *et al.*, 2002). During oogenesis, choriogenins are produced in the liver as a response to oestrogens and released into the bloodstream to be incorporated into the egg envelope (Lee *et al.*, 2002; Arukwe and Goksøyr, 2003). In the Japanese medaka, *Oryzias latipes*, the respective precursors of choriogenin subunits contain a domain characterised by aligned cysteine residues, which is frequently found in glycoproteins of the fish egg envelope (Murata *et al.*, 1994; Grau *et al.*, 1996; Sarasquete *et al.*, 2002a,b).

The swordfish is an important commercial species with an extensive seasonal migration and a circumglobal distribution. It is a gonochoristic species, with females attaining larger sizes than males, and it lives to at least 9 years of age (Rey, 1988). Moreover, in the Mediterranean areas this species often suffers stress due to lipophilic contaminants (Fossi *et al.*, 2001), which could be analysed and characterised by using specific cell biomarkers. The present study investigates the presence and cellular distribution of carbohydrates, glycoconjugates, metal binding proteins, aminoacids, lipids, thyroid hormones and cations on oocytes of the swordfish *Xiphias gladius*

by using a battery of histochemical and immunohistochemical techniques.

## MATERIAL AND METHODS

### Histological procedures

Adult specimens of the swordfish *Xiphius gladius* were caught by harpoon from traditional Sicilian “passerella” fishing boats during the spawning period (June-August) in the Strait of Messina (Sicily, Italy).

Fragments of the liver and ovary were dissected and fixed in Bouin (picric acid, formaldehyde, acetic acid, 15/5/1, v/v/v) for 24 hours and conserved in 70% ethanol, dehydrated through graded alcohols, cleared in xylene and embedded in paraffin. Serial sections (6-7 µm thick) were cut and mounted in gelatinised slides. To identify lipid inclusions, some samples were preserved in 70% ethanol, treated with 1% osmium tetroxide and 2.5% potassium dichromate for 8 h, washed in running water and dehydrated before the wax embedding procedure (Luna, 1968). Haematoxylin-Eosin and Haematoxylin-VOF polychromic stains were performed according to Gutiérrez (1990) and Sarasquete and Gutiérrez (2005).

### Histochemical techniques

Cytochemical techniques were performed to analyse carbohydrates (periodic acid-Schiff [PAS], diastase-PAS, KOH-PAS and Alcian Blue pH 2.5, 1 and 0.5), and to identify proteins in general (Bromophenol blue), proteins rich in lysine (Ninhydrin-Schiff), tyrosine (Hg-sulphate-sulphuric acid-sodium nitrate), tryptophan (p-dimethylaminobenzaldehyde) and arginine (1,2 naphthoquinone-4-sulphonic acid salt sodium). Proteins containing cysteine and cystine (–SH- and –SS- groups) were detected by means of ferric ferricyanide (Fe III) and thioglycolate reduction methods. Calcium was detected with the Alizarin Red technique, and iron was stained with the Prussian Blue (Fe<sup>3+</sup>) and Turnbull-Blue (Fe<sup>2+</sup>) techniques. All histochemical methods are described by Martoja and Martoja-Pierson (1970), Pearse (1985) and Bancroft and Stevens (1990).

### Lectin histochemistry

To analyse glycoconjugates, sections were treated with 0.3% hydrogen peroxide for 15 minutes (to in-

TABLE 1. – Peroxidase-conjugated lectins (HRP-lectin) used for histochemical characterisation of sugar residues of glycoconjugates

Lectin	Concentration (µg/ml)	Carbohydrate binding sugar
Group 1.		
Glucose and manose		
Con A ( <i>Canavalia ensiformes</i> )	25	α-D-Man/D-Glc
Group 2.		
N-Acetylglucosamine		
WGA ( <i>Triticum vulgaris</i> )	20	β-D-GlcNAc/sialic acids
Group 3.		
Galactose and N-acetylgalactosamine		
ECA ( <i>Erythrina cristagalli</i> )	25	β-Gal-GlcNAc
PNA I ( <i>Arachis hipogea</i> )	25	β-D-Gal(1-3)-GalNAc
VVA ( <i>Vicia villosa</i> )	25	α or β-D-GalNAc
DBA ( <i>Dolichus biflorus</i> )	20	α-D-GalNAc
Group 4.		
L-Fucose		
TP ( <i>Tetragonolobus purpureus</i> )	20	α-L-Fuc
UEA I ( <i>Ulex europaeus</i> )	25	-L-Fuc

hibit endogenous peroxidase) in Tris buffered saline (TBS) at pH= 7.2. The sections were then incubated for 1 to 4 hours at room temperature with different horseradish peroxidase-conjugated lectins (HRP-lectin conjugated) dissolved in TBS at the concentrations indicated in Table 1. After three washes in TBS, peroxidase activity was visualised with TBS containing 0.05% 3,3' diaminobenzidine tetrahydrochloride (DAB) and 0.015% hydrogen peroxide. Sections were washed in running tap water for 10 minutes, dehydrated, cleared and mounted in Eukitt. Controls were: omission of the respective lectin; substitution of lectin-HRP conjugated by TBS; and treatments with different enzymes (neuraminidase Type V, β-galactosidase Grade VI, α-mannosidase Type III, β-N-acetylglucosamine, β-N-acetylgalactosamine and L-fucosidase). Controls for lectin specificity included: 1) substitution of the substrate medium with buffer without lectin; and 2) incubation with each lectin in the presence of its hapten sugar (0.2-0.5 M in Tris buffer). Lectins and enzymes were purchased from Sigma Chemical Co. St Louis, MO, USA.

### Immunohistochemical procedure

Endogenous peroxidase activity was blocked in the dark with 3% hydrogen peroxide in Coons buffer with Triton X-100 (CBT- 0.01M veronal, 0.15M NaCl, 0.1% Triton X-100) for 15 min. Non-specific protein binding to sections was blocked with 0.5%

(wt/vol) bovine serum albumin (BSA) in CBT for 30 min. T<sub>4</sub> (thyroxine) and T<sub>3</sub> (triiodothyronine) primary monoclonal antibodies (Fitzgerald Industries) diluted in CBT/0.5% BSA (T<sub>4</sub> antiserum diluted 1:50 and T<sub>3</sub> antiserum diluted 1:25) and with anti-seabream Vg, anti-salmon Zrp and anti-cod Mt primary polyclonal antibodies (Biosense laboratories, Bergen, Norway) were used. Antibodies were diluted 1:250 to 1:500 and incubations were performed overnight in a humidified chamber at room temperature. Sections were washed in CBT and incubated for 1 h at room temperature with goat anti-mouse IgG peroxidase conjugated for monoclonal primary antibodies and with goat anti-rabbit IgG-peroxidase conjugated for polyclonal primary antisera (Sigma, St Louis, MO, USA), with a 1:1500 dilution. Sections were washed again in CBT and in Tris-HCl (0.05 M, pH 7.4). Peroxidase activity was visualised in the dark with 0.025% 3,3'-diamino benzidine tetrahydrochloride/DAB (Sigma) in Tris-HCl 0.05 M, pH 7.6 containing 0.5% hydrogen peroxide. The stained sections were mounted in an aqueous mounting medium (Aquatex, Merck). To confirm the specificity of immunostainings, negative controls were performed by replacing the primary antibody with pre-immune serum (from the same animals from which the antiserum was obtained) or BSA, and by omission of primary and secondary antibodies.

Results were visualised using a Diaplan Leitz light microscope equipped with an Insight Spot digital camera.

## RESULTS

### Histochemical characteristics of oocytes of the swordfish *Xiphias gladius*

Oocytes at different stages of development (oogonia, previtellogenic, vitellogenic, maturing, atretic and post-ovulatory follicles) were detected in most *X. gladius* ovaries studied (Fig. 1A-J). A high percentage of vitellogenic and maturation oocytes, and the presence of atretic oocytes and especially of post-ovulatory follicles, were indicative of advanced vitellogenesis, maturation and spawning phases.

The histochemical results are summarised in Table 2. Yolk granules showed orange G (Haematoxylin-VOF) or eosin affinities (Haematoxylin-Eosin) and lipid globules appeared unstained with

both morphological dyes. Neutral lipids (globules or vacuoles in paraffin sections) showed a strong affinity towards osmium tetroxide. Lipid globules increased in number and size and gradually fused and coalesced during the maturation phase (Figs. 1, 2A-C).

Yolk granules reacted weakly with PAS and diastase-PAS (presence of neutral glycoproteins/glycolipids and absence of glycogen) (Fig. 2D) and they were strongly stained with protein techniques, especially those rich in -SH- and -SS- groups (i.e. cysteine and cystine) (Fig. 2E-H) and unstained with Alcian Blue techniques (AB, pH: 0.5, 1 and 2.5). Intergranular cytoplasm contained proteins rich in different amino acids, especially those rich in tyrosine, tryptophan, cysteine and cystine (Figs. 2d-h, 3a-c). Intergranular cytoplasm also contained iron (Fe<sup>3+</sup>) and calcium. Cortical alveoli were composed of carbohydrates (carboxylated and neutral glycoproteins) and proteins rich in -SH and -SS- groups exclusively.

In *X. gladius*, the zona radiata was mainly proteinaceous in nature. This egg envelope showed a strong acidophilia/eosinophilia and was composed of an internal part (ZrI) with an affinity for acid fuchsin and an external part (ZrE) with a tinctorial affinity for light green (H-VOF stain). Proteins in general and proteins rich in amino acids (i.e. arginine, cystine, cysteine and tryptophan) were detected in the zona radiata. The ZrE showed a stronger PAS-positive reaction (neutral glycoproteins) than the ZrI, which contained proteins rich in disulphide (-S-S-) groups (Figs. 2D-H, 3A-C).

### Lectin histochemistry

Glycoconjugates containing sugar residues were detected in cell structures of vitellogenic and mature swordfish oocytes (Figs. 3D-F, 4A-H; Table 3). Intergranular cytoplasm and yolk granules were stained, with variable intensity, by DBA ( $\alpha$ -GalNAc), ConA ( $\alpha$ -Man,  $\alpha$ -GlcNAc) VVA (GalNAc), UEA I ( $\alpha$ -L-Fuc), WGA ( $\beta$ -GlcNAc, sialic acid) and ECA ( $\beta$ -Gal, GalNAc) lectins. Yolk granules showed a specific affinity for PNA I ( $\beta$ -Gal, GalNAc) and TP( $\alpha$ -L-Fuc). Cortical alveoli contained carboxylated and neutral glycoproteins and lectin techniques detected N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, galactose and sialic acid groups. The zona radiata was weakly reactive to WGA and TP (Table 3).



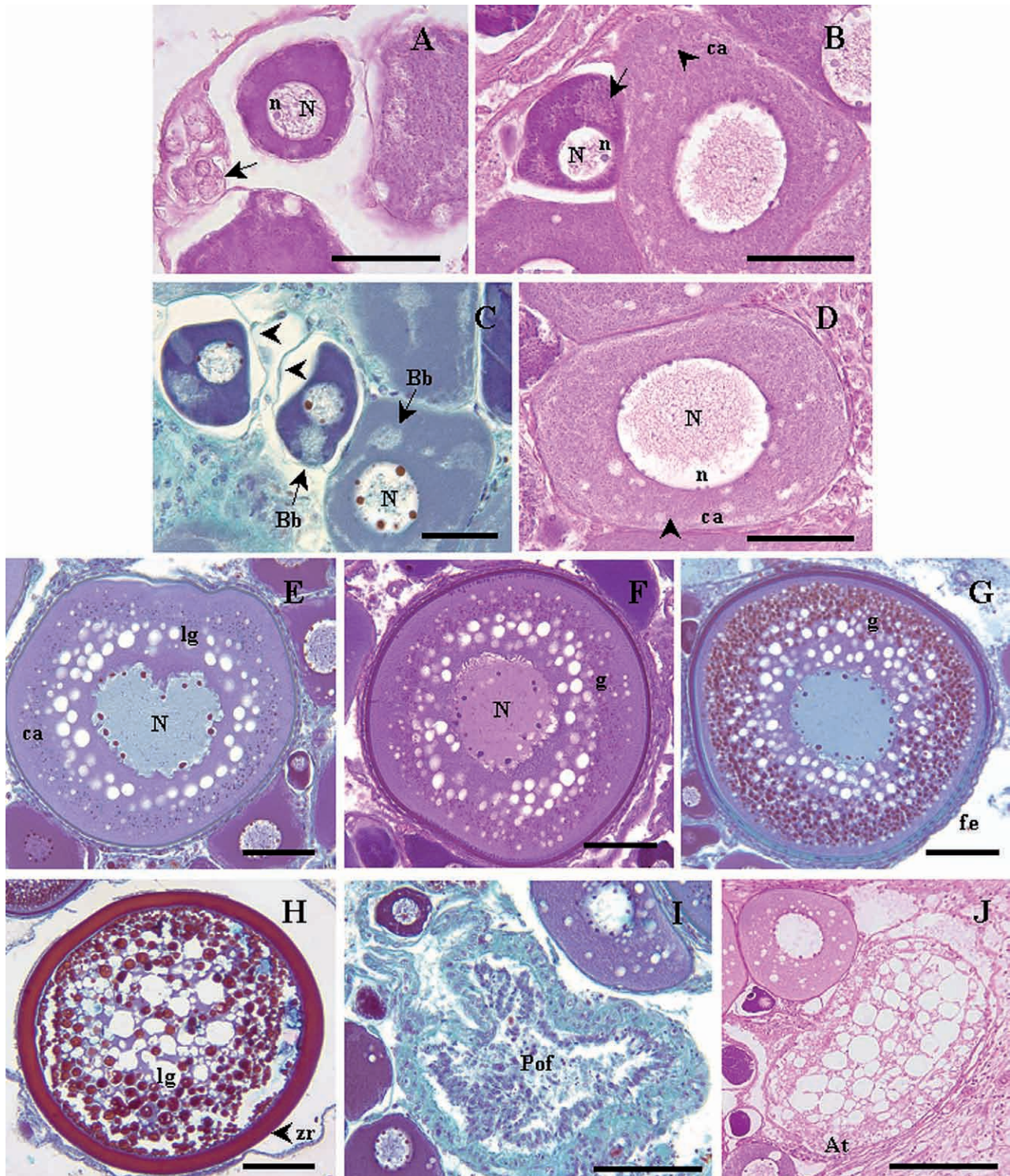


FIG. 1. – Photomicrographs of swordfish ovaries at different maturation stages. A, cluster of oögonia adjacent to the oocyte chromatin nucleolus stage (arrow). B, oocyte in advanced chromatin nucleolus stage showing cortical alveoli (ca) (arrowhead) in association with early perinucleolus stage oocytes (arrow, Balbiani bodies). C, early perinucleolus stage showing Balbiani bodies (Bb) and a layer of pavement epithelial cells surrounding the oocytes (arrowheads). D, early lipid oocyte stage showing several cortical alveoli (ca). E, late lipid oocyte stage showing lipid globules (lg) and cortical alveoli (ca). F and G, early vitellogenic stage showing yolk granules (g) and developed follicular envelope (fe). H, oocyte in late vitellogenic stage showing coalescence of lipid droplets (lg) and yolk granules. I, postovulatory follicle (Pof). J, atretic oocyte (At). n: nucleolus; N: nucleus. Scale bars represent 100 µm.



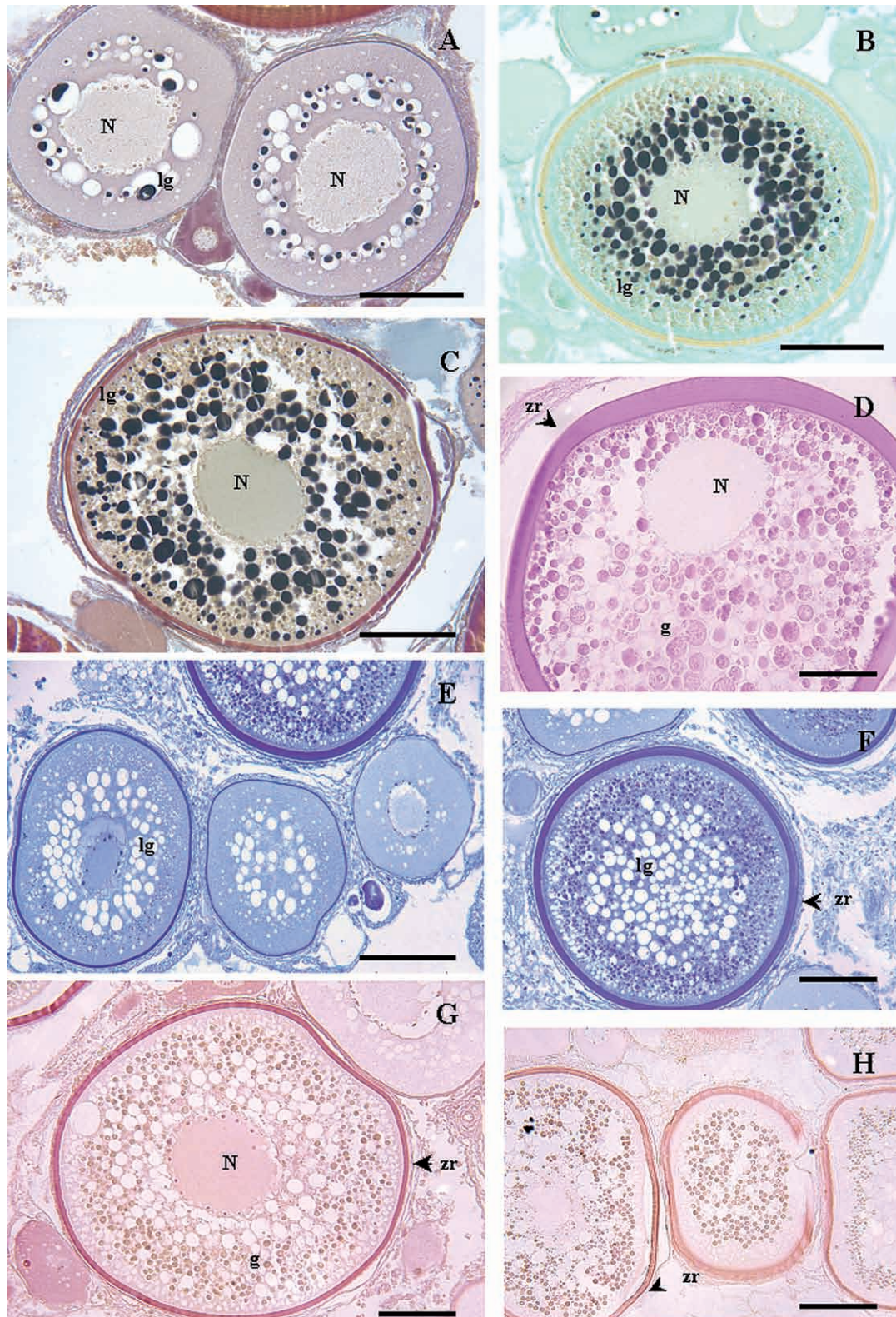


FIG. 2. – Early and late vitellogenic oocytes showing neutral lipids and glycoconjugates. A, B and C, sequence of active lipidogenesis showing increased lipid globules (lg) around nucleus and lipid coalescence in advanced stages; affinity by osmium tetroxide. D, neutral glycoproteins within the yolk granules (g) and zona radiata (zr) in postvitellogenic oocyte starting the maturation phase. Note the migration of the nucleus and the coalescence of lipid globules and yolk granules (PAS reaction). E and F, bromophenol blue technique (general proteins) showing strong staining in the zona radiata (zr) layer. Presence of proteins rich in: G, arginine; and H, tyrosine in zona radiata (zr) and yolk granules (g). lg: lipid globules; N: nucleus. Scale bars represent 100 μm.

TABLE 2. – Histochemistry of the *X. gladius* oocytes.

	Cortical alveoli	Oil globules	Yolk granules	Intergranular cytoplasm	Follicular envelope	Zona radiata
PAS	+/-	-	+	-	+/-	++
Diastase-PAS	+/-	-	+	-	+/-	++
KOH-PAS	+	-	+	++	+/-	++
AA 2.5	+/-	-	-	+	+/-	-
AA 1	-	-	-	-	-	-
AA 0.5	-	-	-	-	-	-
Proteins	-	-	+++	++	++	+++
Lysine	-	-	+/-	-	+/-	+/-
Arginine	-	-	+/-	+/-	+/-	+++
Tyrosine	-	-	+	+	-	+
Tryptophan	-	-	+	+	-	++
Cysteine	-	-	++	+	+	++
Cystine	-	-	++	+	+	+++
Neutral lipids	-	+++	-	-	-	-
Calcium	-	-	+	+/-	+/-	++
Iron-Fe2+	-	-	-	-	-	-
Iron-Fe3+	-	-	+/-	+/-	-	-

Intensity of reaction: +/-: weak; +: moderate; ++: strong; +++: very strong.

TABLE 3. – Distribution of sugar residues of glycoconjugates in *X. gladius* oocytes

Lectins	Cortical alveoli	Oil globule	Yolk granules	Intergranular cytoplasm	Follicular envelope	Zona radiata
DBA	++	-	+/-	++	++	-
ConA	-	-	+/-	++	++	-
UEA I	-	-	+	+	++	-
WGA	+	-	+	+	-	+
ECA	++	-	++	+	-	-
PNA I	+/-	-	+/-	-	++	-
TP	-	-	+/-	-	++	+/-
VVA	++	-	+/-	++	++	-

Intensity of reaction: +/-: weak; +: moderate; ++: strong.

TABLE 4. – Immunohistochemical localisation of Vg, Zrp, Mt and thyroid ( $T_3$ ,  $T_4$ ) hormones in oocytes of the swordfish *X. gladius*.

	Cortical alveoli	Oil globules	Yolk granules	Intergranular cytoplasm	Follicular envelope	Zona radiata
Vitellogenin/Vg	-	-	++	+	-	-
Zona radiata protein/Zrp	-	-	-	-	-	+++
Metallothionein/Mt	++	-	+/-	+	++	-
$T_3$	+	-	-	+/-	++	-
$T_4$	-	-	-	+/-	+	-

Intensity of reaction: +/-: weak; +: moderate; ++: strong; +++: very strong.

### Immunohistochemical distribution of Mt, Vg and Zrp, $T_3$ and $T_4$

Immunohistochemical localisation of Mt,  $T_3$ ,  $T_4$ , Vg and Zrp in oocytes of the swordfish *X. gladius* is summarised in Table 4. Cortical alveoli and follicular envelopes showed strong Mt immunostaining and weak or moderate immunoreactivities were detected in cytoplasm and yolk granules (Fig. 5A-C) (Table 4). Calcium was strongly positive in yolk granules and zona radiata and a weak alizarin red staining was detected in both oocyte cytoplasm and the follicular envelope (Fig 5d). Mt was also detected in the liver,

within the hepatocytes and vascular system of both males and females.

Cortical alveoli showed a moderate anti- $T_3$  immunoreactivity, whereas the follicular envelope and intergranular cytoplasm showed variable  $T_3$  and  $T_4$  immunostaining. However, no immunoreactivity was detected in oil globules, yolk granules and zona radiata using both thyroid primary antisera (Table 4; Fig 5 E, F).

*X. gladius* oocytes showed positive Vg immunostaining. A weak staining was detected in scarce yolk granules present within the ooplasm during the early vitellogenesis stage, whereas late vitellogenic



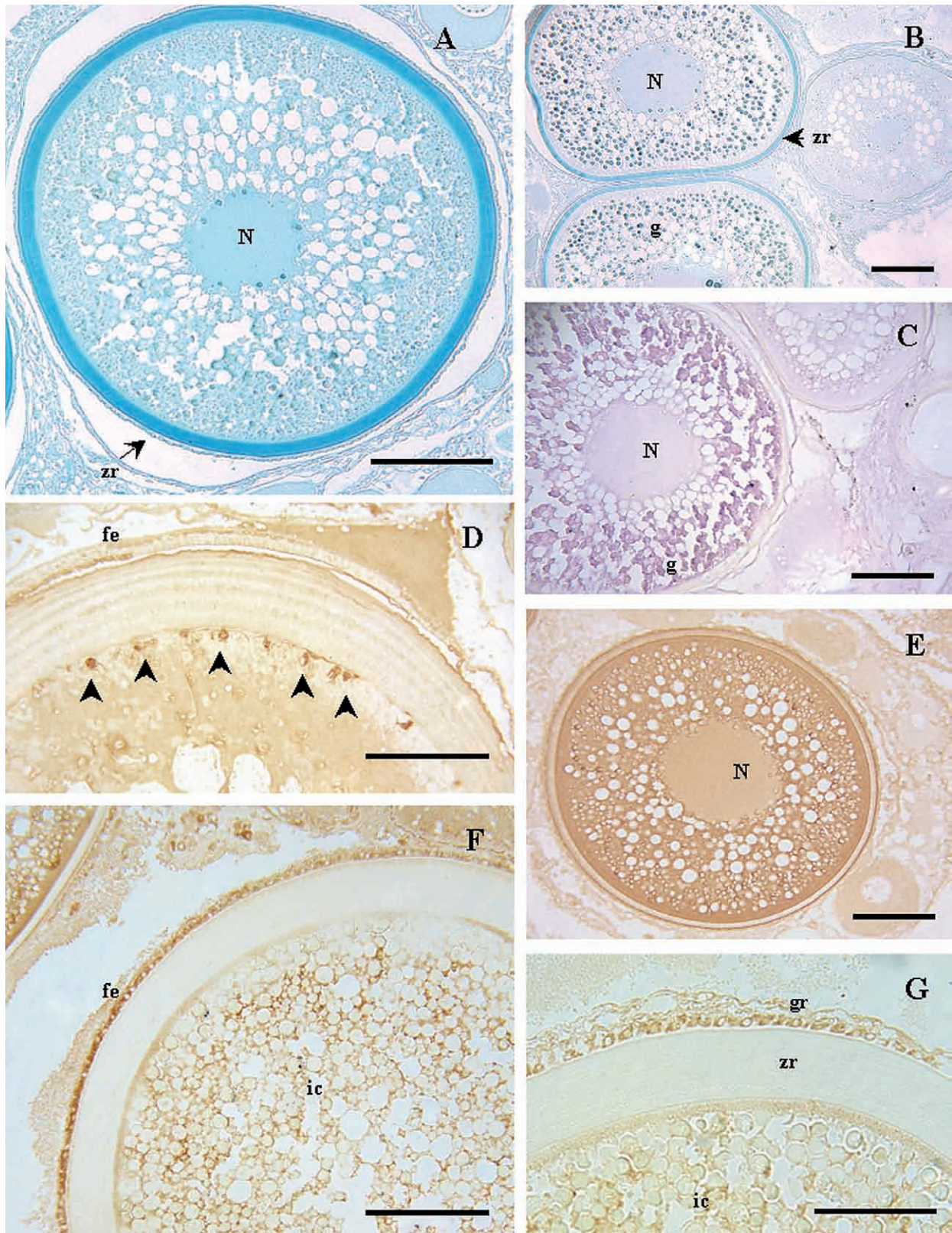


FIG. 3. – Early and late vitellogenic oocytes showing protein-rich: A, tryptophan; B, cysteine; and C, cystine within yolk granules (g), follicular envelope and zona radiata (zr). D and E, moderate reaction of glycoconjugates containing GalNAc sugar residues in the cortical alveoli (arrowheads) and within intergranular cytoplasm respectively. F and G, moderate reaction of glycoconjugates containing Man and/or Glc sugar residues in the intergranular cytoplasm (ic) and granulosa cell layer (gr). N: nucleus; fe: follicular envelope; zr: zona radiata. Scale bars represent 50 μm.



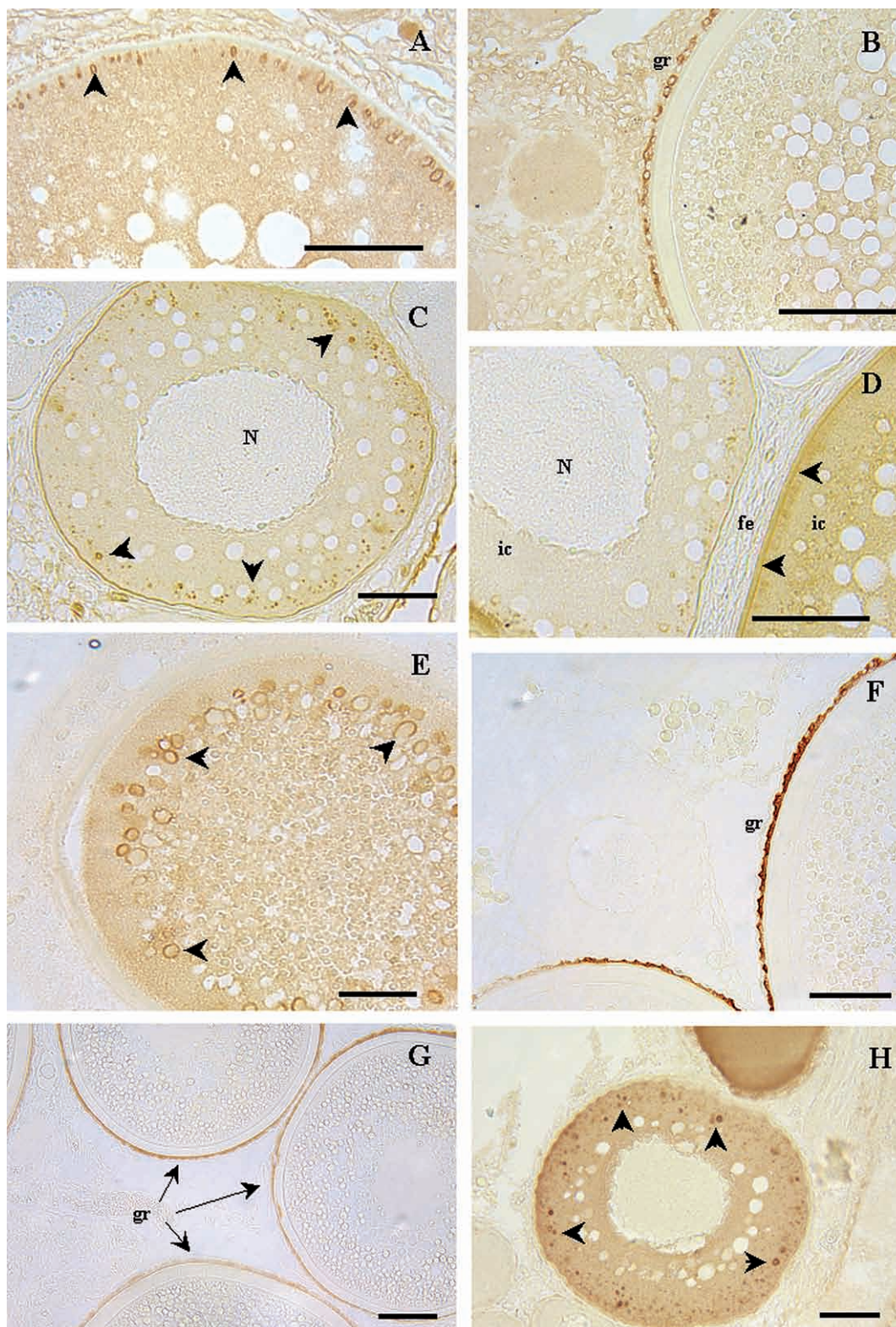


FIG. 4. – Early and late vitellogenic oocytes showing presence of glycoconjugates. A and B, moderate to strong reaction of glycoconjugates containing Fuc sugar residues in cortical alveoli (arrowheads) and granulosal cell layer (gr). C and D, glycoconjugates containing GlcNAc and/or sialic acids sugar residues in cortical alveoli (arrowheads) and intergranular cytoplasm (ic). Note the absence of staining in the follicular envelope (fe) and zona radiata. E, weak reaction of glycoconjugates containing Gal and/or GlcNAc sugar residues in the cortical alveoli (arrowheads). F and G, strong reaction of glycoconjugates containing Gal and/or GalNAc and L-Fuc in the follicular cell layer (granulosa cells: gr). H, glycoconjugates containing GalNAc sugar residues in cortical alveoli (arrowheads) and intergranular cytoplasm. N: nucleus. Scale bars represent 50  $\mu$ m.



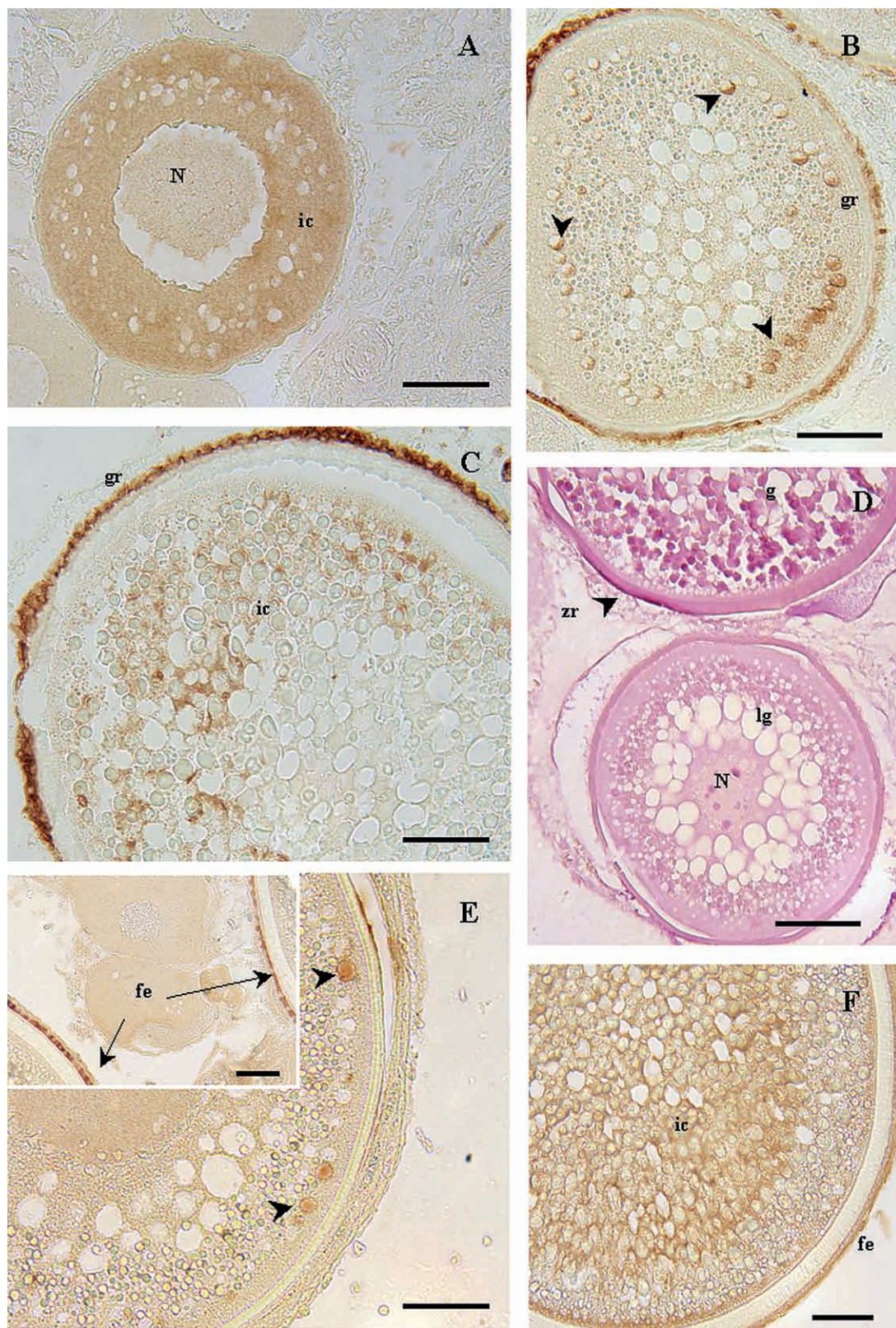


FIG. 5. – Oocytes at different maturation stages showing moderate immunoreaction by metallothionein, thyroid hormones and calcium. A, B and C, anti-Mt immunoreactivity within the intergranular cytoplasm (ic), granulosa cell layer (gr) and cortical alveoli (arrowheads). D, yolk granules (g), intergranular cytoplasm and zona radiata (zr) containing calcium. Alizarin red technique. E and F, anti-T<sub>3</sub> and T<sub>4</sub> immunoreactivities in follicular envelope (fe), intergranular cytoplasm (ic) and cortical alveoli (arrows); (T<sub>3</sub>) intergranular cytoplasm (ic) and (T<sub>4</sub>) follicular envelope (fe). lg: lipid globules; N: nucleus. Scale bars represent 50  $\mu$ m.



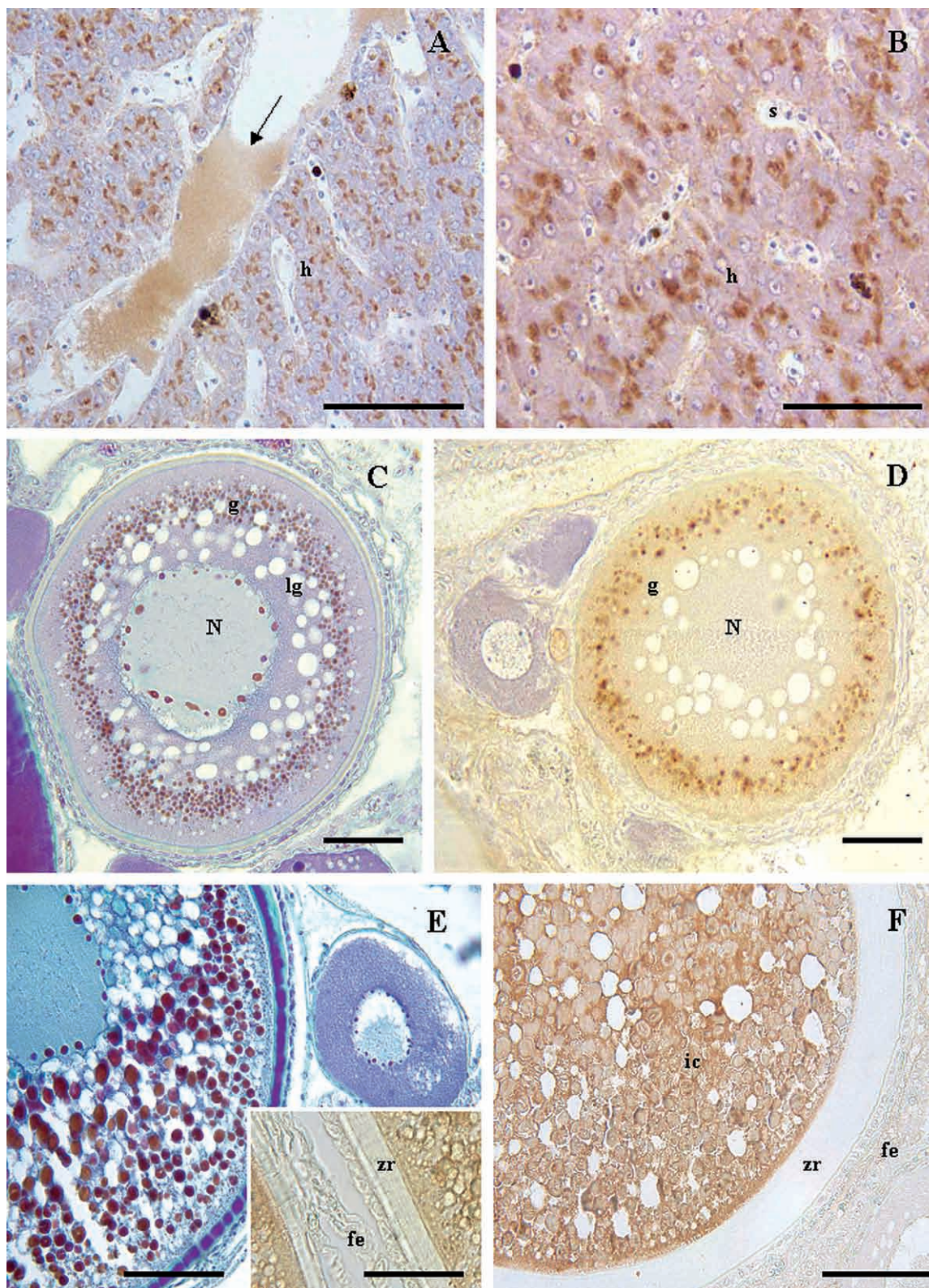


FIG. 6. – Immunohistochemical localisation of vitellogenin (Vg) in liver and ovary of *X. gladius* showing: A and B, strong Vg immunostaining within the cytoplasm of hepatocytes (h) concentrated close to the canalicular border, as well as surrounding the sinusoidal endothelia. Note plasma Vg immunostaining (arrow), suggesting an active Vg releasing from hepatocytes in the blood stream. C and D, Vg immunostaining in yolk granules (g) of early stage vitellogenic oocyte. E, numerous yolk granules and lipid globules in the vitellogenic oocyte. F, moderate or strong immunostaining intensity for Vg in the yolk granules and intergranular cytoplasm (ic). Note absence of staining in the follicular envelope (fe) and zona radiata (zr) (Figure E insert). N: nucleus; lg: lipid globules. Scale bars represent 100 µm.



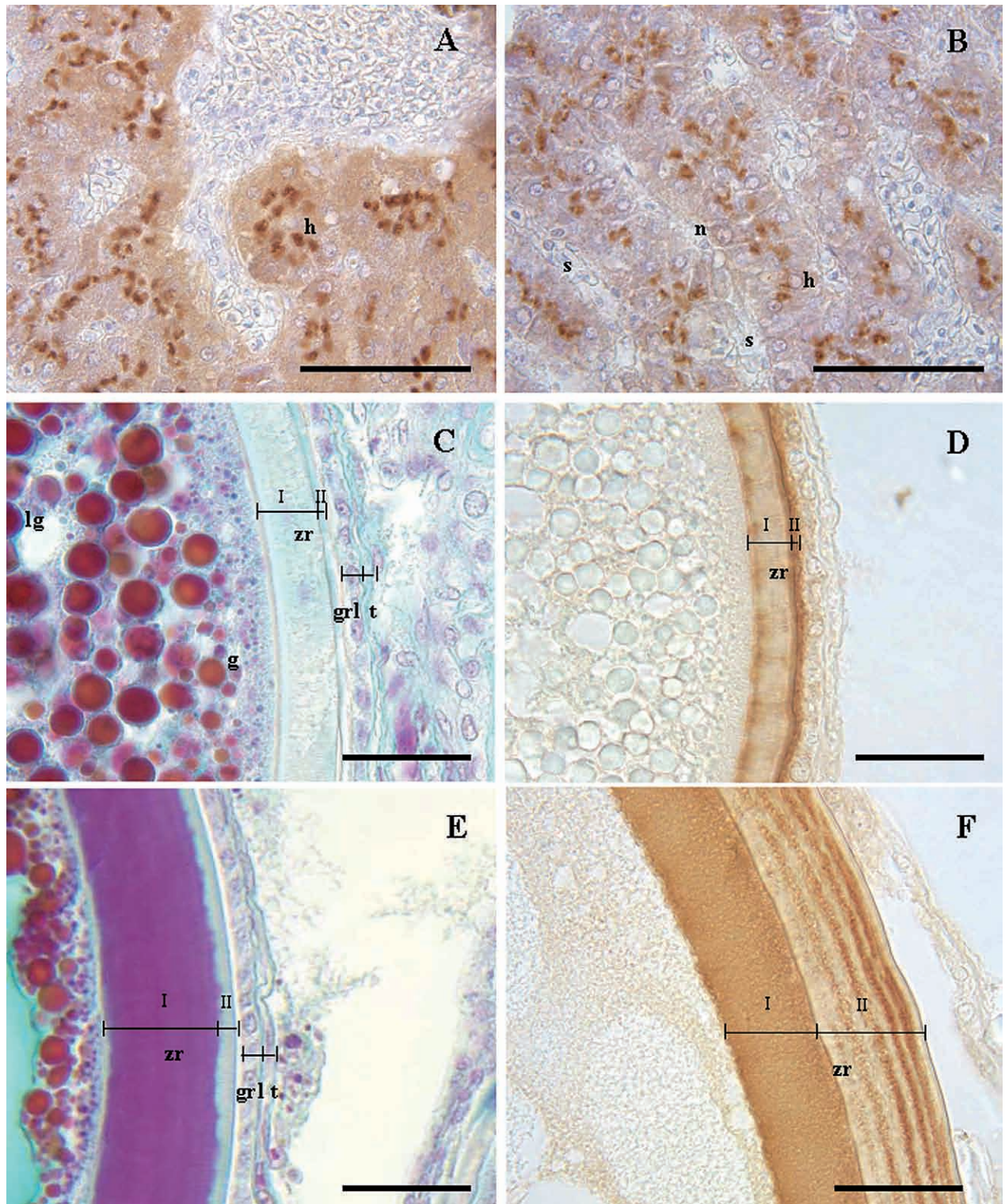


FIG. 7. – Immunohistochemical localisation of Zona radiata protein (Zrp) in liver and ovary: A and B, very weak Zrp immunostaining spread along the whole cytoplasm of the hepatocytes (h) and strong Zrp staining concentrated close to the canaliculi border. C, vitellogenic oocyte showing numerous acidophilic yolk granules (g) (orange G affinity) and lipid globules (lg) (empty vacuoles). The zona radiata (zr) shows a bipartite structure constituted by a thinner external zone (zrE/II) and a less acidophilic internal radiata zone (zrI/I). The follicular envelope was divided into two differentiated layers: the granulosa (grl) and a thin external cell layer or theca (t). D, anti-Zrp immunoreactivity detected in the external radiata zone (zrE/II) and less intense immunostaining in the internal zone (I). E, late stage vitellogenic oocyte showing a striated zona radiata (zr). F, strong anti-Zrp immunoreactivity in the internal homogeneous zone (I) and moderate Zrp staining in the longitudinally-striated external zone (II). No Zrp immunostaining was detected in the follicular envelope. n : hepatocyte nucleus; s : sinusoids; grl : granulosa layer; t : theca layer. Scale bars represent 100 µm.



oocytes displayed a strong Vg staining in numerous yolk granules located within the ooplasm. Moderate Vg immunostaining was detected in intergranular cytoplasm of the swordfish vitellogenic oocytes. The liver also showed positive Vg immunostaining, especially evident in the plasma content of the vascular system, in the cytoplasm of hepatocytes, and surrounding the canalicular border of hepatic cells. However, no Vg immunostaining was detected in the sinusoidal endothelium (Fig. 6A-F).

Both the ZrE and ZrI layers of the zona radiata showed a strong Zrp immunoreactivity, with higher staining in the internal layer than in the ZrE. The liver also showed Zrp immunoreactivity, which was located as condensed granules within the cytoplasm and along the canalicular border of parenchymal cells (Fig. 7A-F; Table 4).

## DISCUSSION

The histological characteristics of the oogenesis of the swordfish *Xiphias gladius* were similar to those previously described by Corriero *et al.* (2004). The ovarian maturation consisted of oocytes at different developmental stages, and especially a high percentage of vitellogenic, maturing, atretic and post-ovulatory follicles, which is typical of a multiple spawner species during the freezing period.

In swordfish oocytes, as in those of other fish species (Selman *et al.*, 1986; Sarasquete *et al.*, 2002 a,b; Motta *et al.*, 2005), cortical alveoli contain a considerable cellular heterogeneity of glycoconjugate sugar residues (i.e. N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, galactose and sialic acid), suggesting that they may exert a primary intracellular role by acting, for example, as carriers or cross-linking agents for the glycosylated alveolar components (Olden *et al.*, 1982). In medaka eggs, Shibata *et al.* (2000) reported the existence of a specific metalloproteinase, the alveolin, also responsible for chorion hardening following fertilisation. The fact that alveoli lectins and glycoconjugates are extruded into the perivitelline space at fertilisation could indicate that they are not an important source of nutrients to the embryos (Yamamoto, 1962), but different glycoconjugates may be involved in sperm and bacteria agglutination (Prokop *et al.*, 1974) or in binding of sperm to the egg surface, as suggested by Kothbauer and Schenkel-Bruner (1974).

It is well known that thyroid hormones play an important role during embryogenesis and organogenesis, and most vertebrates are unable to grow and reach their normal adult form without them (Liu *et al.*, 2000; Power *et al.*, 2001). The presence of thyroid hormones in fish eggs prior to hatching has been reported in several fish species and these are presumably of maternal origin (De Jesus *et al.*, 1991; Lam, 1995; Yamano, 2005). Recently, in *Solea senegalensis* larvae development, Ortiz-Delgado *et al.* (2006) detected T<sub>3</sub> and T<sub>4</sub> immunostaining in the yolk sac matrix at hatching. Interestingly, a weak presence of thyroid hormones/T<sub>3</sub> and metallothionein/Mt was detected in swordfish cortical alveoli. Effects of the ovarian fluids as hormones, which act through the hypothalamic-pituitary axis to stimulate the activation of the thyroid gland, were pointed out (Finn, 2007). Moreover, according to Finn, ovarian fluids can act as an important trap for cations, being also the major barrier to diffusion of gases and solutes.

The zona radiata of swordfish oocytes, also known as the chorion, is immunostained with anti-salmon Zrp. This acellular protein egg envelope contains calcium and is composed of neutral mucopolysaccharides and especially of proteins rich in cysteine and cystine, which suggests that most of the proteinaceous material of this multilamellar layer is determined by the formation of disulphide bonds (Hagenmayer, 1973; Gutiérrez *et al.*, 1985; Sarasquete *et al.*, 1993, 2002 a, b; Grau *et al.*, 1996; Mayer *et al.*, 1988). In *Salmo salar* radiata envelopes, Hamor and Garside (1973) detected proteins, mucopolysaccharides, phospholipids, cholesterol, nucleic acids and oxidative enzymes. Studies in other fish species (Tesoriero, 1977) have shown that the external layer of the zona radiata was rich in polysaccharides, whereas the inner layer was rich in proteins. In *Carassius auratus* (Khoo, 1979) and *Dicentrarchus labrax* (Mayer *et al.*, 1988), polysaccharides adhere to the outer layer which might contribute to the adhesion of the eggs. However, in *Siganus rivulatus* eggs, the chorion consists exclusively of proteins (Lahnsteiner and Patzner, 1999).

The liver of swordfish females showed moderate to strong Zrp, Vg and Mt immunoreactivities. In *X. gladius* oocytes, Vg was detected in yolk granules and intergranular cytoplasm but not in the follicular envelope. According to Abraham *et al.* (1984) the follicular cells are not involved directly in the transfer of exogenous material into the oocytes. However, in other fish species Vg was present in the follicular

envelope (Susca *et al.*, 2001; Sarasquete *et al.*, 2002 a, b). Vg immunostaining was also strongly detected in hepatocytes, especially in the canalicular border, and it was weakly detected in the hepatic vascular system. Hepatocytes have previously been identified as the site of exogenous vitellogenesis protein synthesis, and the vascular system has been identified as the transferral route between the liver and ovary (Wallace, 1978). Vg immunoreactivity was detected in the liver of brown trout, *Salmo trutta* females, and no staining was observed in the liver of males (Wahli *et al.*, 1998). Interestingly, as indicated previously in Mediterranean swordfish specimens (Fossi *et al.*, 2001, 2007, Desantis *et al.*, 2005), we observed positive Vg immunoreactivity in the liver of some male specimens (data not shown), which also presented high plasma oestradiol, Vg and Zrp concentrations, as well as high endocrine disrupting chemical levels (DDTs, PCBs, PAHs, etc) in the liver, gonads and plasma (Mori *et al.*, 2005). The capacity of the liver of fish males to synthesise Zrp and Vg after exposure to natural estrogens or man-made chemicals has been widely reported (Oppen-Berntsen *et al.*, 1992, 1999, Fossi *et al.*, 2001, 2007; Desantis *et al.*, 2005).

In the reproductive cycle of vertebrate and invertebrate species, Mt-like proteins play an important physiological role during vitellogenesis, because these proteins participate in homeostasis of zinc and copper (Webb, 1987; De Prisco *et al.*, 1991; Banks *et al.*, 1999), and Mt could also participate in the regulation of Vg synthesis. In *X. gladius* oocytes, Mt was strongly detected in the follicular layer (granulosa cells), cortical alveoli and intergranular cytoplasm, and was weakly detected in yolk granules of the vitellogenic oocytes, as well as in the liver of both male and female swordfish specimens. In fish species, Olsson *et al.* (1989) and Carpane *et al.* (1994) suggested that zinc is an essential macronutrient for oocyte development. Banks *et al.* (1999) in channel catfish indicated the importance of Mt-like proteins in zinc regulation during exogenous vitellogenesis and fast oocyte growth.

In conclusion, the histochemical composition of oocytes of the swordfish *Xiphias gladius* (cytoplasm, cortical alveoli, yolk and egg envelopes) can indicate specific physiological reproductive functions. Sugar residue of glycoconjugate cortical alveoli contents ( $\beta$ -D-GlcNAc, sialic acids,  $\alpha$ -D-GalNAc,  $\alpha$  or  $\beta$ -D-GalNAc,  $\beta$ -D-Gal(1-3)-GalNAc), which are discharged into the perivitelline space at fertilisation, may be involved in chorion hardening, binding

of sperm to the egg surface, and polyspermy block after gamete fusion, among other functions. As in most fish species, globules consisting of neutral lipids exclusively and glycopospholipoprotein yolk granules are the major energetic and nutritional resources respectively of eggs, embryos and larvae. During swordfish oogenesis, from a histochemical point of view, possible interactions of Mt and Vg synthesis could be suggested. Both complex proteins are localised within the yolk granules and intergranular cytoplasm during the vitellogenesis and maturation phases. Finally, immunohistochemical localisation of thyroid hormones in the cortical alveoli, intergranular ooplasm and follicular envelope could indicate their maternal origin, as well as an essential role of  $T_3/T_4$  during swordfish oogenesis.

Studies are currently being performed to analyse inorganic and organic contaminants, xenobiotic induction of molecular, biochemical and cell biomarkers (Vg, Zrp, Mt, oestrogens, thyroid hormones, etc.), and presence of molecular and histopathological disorders in swordfish males and females from several Mediterranean areas.

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